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Review Article

How reliable and robust are current biomarkers for copper status?

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Cu is an essential nutrient for man, but can be toxic if intakes are too high. In sensitive populations, marginal over- or under-exposure can have detrimental effects. Malnourished children, the elderly, and pregnant or lactating females may be susceptible for Cu deficiency. Cu status and exposure in the population can currently not be easily measured, as neither plasma Cu nor plasma cuproenzymes reflect Cu status precisely. Some blood markers (such as ceruloplasmin) indicate severe Cu depletion, but do not inversely respond to Cu excess, and are not suitable to indicate marginal states. A biomarker of Cu is needed that is sensitive to small changes in Cu status, and that responds to Cu excess as well as deficiency. Such a marker will aid in monitoring Cu status in large populations, and will help to avoid chronic health effects (for example, liver damage in chronic toxicity, osteoporosis, loss of collagen stability, or increased susceptibility to infections in deficiency). The advent of high-throughput technologies has enabled us to screen for potential biomarkers in the whole proteome of a cell, not excluding markers that have no direct link to Cu. Further, this screening allows us to search for a whole group of proteins that, in combination, reflect Cu status. The present review emphasises the need to find sensitive biomarkers for Cu, examines potential markers of Cu status already available, and discusses methods to identify a novel suite of biomarkers.

Copper status: Copper excess: Copper deficiency: Public health: Biomarkers

Copper essentiality and toxicity

Cu is an essential micronutrient for man, but potentially toxic when intake levels are too high. As a transition metal, it takes part in a variety of biological reduction and oxidation (redox) processes. This makes it important as a cofactor of many redox enzymes, but Cu overload can also lead to participation in the Fenton-type redox reaction and cause oxidative damage to cells[1].

Cu deficiency, especially during pregnancy, has widespread effects. It can result in impaired heart and blood vessel development and in brain malformation in the fetus[2–4]. Low Cu levels have been linked with bone malformation during development[5,6], and may contribute to the risk of developing osteoporosis later in life[7,8]. A decrease in collagen stability[9] and impaired melanin synthesis can also be consequences of low Cu status[10,11]. Cu deficiency has also been associated with a weakened immune system and an increase in infections (for a review, see Bonham *et al.*[12]), with a decline in cardiovascular health (for a review, see Uriu-Adams & Keen[1]) and adverse alterations in cholesterol metabolism[13–15]. Further, oxidative stress is a consequence of Cu deficiency[1], and – although controversial – low plasma Cu has been linked with a faster decline in cognitive ability in Alzheimer's disease[16,17]. Finally, Cu deficiency affects the metabolism of other trace elements; most notably it perturbs Fe mobilisation and can cause secondary Fe deficiency[18]. Because of the complexity and variety of the symptoms, it is often difficult to diagnose Cu deficiency, rather than other nutritional deficiencies.

Abbreviations: CCO, cytochrome C oxidase; CCS, Cu chaperone for superoxide dismutase; Cp, ceruloplasmin; DAO, diamine oxidase; PAM, peptidylglycine α-amidating mono-oxygenase; SOD, superoxide dismutase; WD, Wilson's disease.

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Cu deficiency is extremely prevalent in malnourished children[19,20]. A recent study in twenty-nine severely or moderately malnourished children demonstrated that all patients were Cu deficient at time of hospitalisation (M Medina and M Araya, unpublished results). Sub-populations at an elevated risk of Cu deficiency include pregnant and lactating women, as well as children during prenatal and neonatal life and during breast-feeding. Disease conditions such as gut absorption problems (coeliac disease, Crohn's disease), or diseases of the immune system (for example, AIDS, all autoimmune diseases) may pose a risk for Cu deficiency. Since many foods high in Cu (liver, oysters, chocolate) are high in fat and not considered part of a healthy lifestyle, Cu deficiency may be a side effect of dieting, especially in young women. Prolonged use of Zn supplements may cause secondary Cu deficiency, as Zn causes an induction of metallothioneins, which then also bind Cu[21]. Further, Zn and Cu compete for intestinal uptake[22–24]. Excessive Zn intake has been shown to cause secondary Cu deficiency in several case studies[25–27], and the consequences of supplementing large segments of the population with moderate or high doses of a single mineral are unknown.

Cu overload is much less likely than Cu deficiency, at least in part since there are very efficient homeostatic mechanisms. Possible targets of Cu overload are mainly the liver and the brain. Cu overload, like Cu deficiency, can lead to cellular oxidative stress. Patients with any liver disease (all forms of hepatitis, alcohol abuse) are sensitive to Cu-related liver toxicity and diabetes has also been linked with high liver Cu[1,28]. There are also subpopulations with a genetic predisposition that may have an elevated risk of Cu accumulation. These include patients suffering from Wilson's disease (WD), caused by mutations in the Cu ATPase ATP7B[29]. ATP7B is a Cu pump responsible for Cu excretion, and WD patients accumulate Cu to toxic levels. WD is an autosomal recessive disorder, and occurs at a frequency of 1 in 30 000.

The prevalence of WD heterozygotes, individuals who carry one WD and one healthy allele of ATP7B, is not known. Heterozygotes appear perfectly healthy, but they may have mild abnormalities in Cu metabolism[30]. These individuals represent a potential public health problem, as they are generally unaware of their condition. At least in principle, they could be more sensitive to high Cu intake, but without good markers of Cu status, no evidence is available.

Copper exposure and copper status in large populations

In man, Cu is supplied in the diet and to varying degrees by drinking water from Cu piping. Good dietary sources of Cu are organ meat (liver), some seafoods (oysters), chocolate and cocoa products, nuts (mainly cashew) and seeds. According to the US National Academy of Sciences (http://math.ucsd.edu/~ebender/Health%20&%20Nutrition/Nutrition/NAS.html), the RDA, or the adequate intake of Cu is 0·9 mg for the general population. The upper limit is 10 mg. Pregnant and nursing women need more Cu, with an RDA of 1 mg and an adequate intake of 1·3 mg. The National Academy of Sciences states that Cu deficiency is common, and experts conclude from the 'EU Voluntary Risk Assessment for copper and copper compounds' that Cu deficiency may be more common than generally thought in Europe.

Despite the realistic possibility of widespread mild Cu deficiency, many regulatory agencies, such as the Californian Environmental Protection Agency, are concerned about potential Cu overexposure (for example, through drinking water). However, Cu status in the population is not easily measured, as neither plasma Cu nor plasma cuproenzymes reflects Cu status[31–33]. Cu status is often estimated by intake and exposure assessments. These are subject to the error inherent to all dietary intake studies and, while suitable for the assessment of macronutrient intake, may not be precise enough to estimate intake of a dietary micronutrient.

The limit for Cu in drinking water according to the WHO is 2 mg/l. This limit is not wholly adopted; for example, the US limit is 1·3 mg/l and many European countries have a limit of 1 mg Cu/l. The reason for this inconsistency is a question of regulatory principles. Local changes in the chemical properties of drinking water (water hardness and pH) can also lead to variations in Cu content of the water.

Regulators are currently not sure what limit makes drinking water 'safe to drink' in all situations. Taken together with the potentially large error of dietary Cu intake assessment, regulators and public health professionals adopt a predominantly conservative approach in Cu-exposure regulation. This approach may not be suitable for an essential trace metal, since a low intake of Cu is as dangerous as a too-high intake.

A good biomarker of Cu is needed, therefore, to monitor and avoid chronic health effects in large populations, and to give an 'early warning' in sensitive populations (infants, pregnant or lactating women, individuals with idiopathic or genetic changes in metabolism, the elderly, disease), before any tissue damage occurs. While it is possible to detect Cu deficiency or excess in their extremes due to ensuing tissue damage (for example, an increase in liver enzymes), it is currently not possible to detect minor but biologically significant variations of Cu status[33,34]. This situation poses an urgent need to develop a 'diagnostic test kit' of Cu status to satisfy regulators and to ensure adequate public health decisions. The biomarker(s) must be able to measure Cu status, including mild deficiency and mild overload, sensitively and specifically.

Copper homeostasis: liver copper and regulation of plasma copper levels

Newly absorbed Cu is transported from the intestine via the portal vein directly to the liver. Here, Cu is stored and redistributed to all other organs[35,36]. Cu status in the body is regulated by both duodenal absorption and/or biliary excretion. As long as exposure is within the homeostatic range, in healthy adults high Cu exposure results in down regulation of Cu uptake in the duodenum, and up regulation of biliary excretion[36–39]. As a result, high Cu exposure or intake does not necessarily cause an equivalent body 'Cu load'. This is one of the reasons why Cu status cannot be estimated by exposure levels. Currently, the only precise indicator of Cu load is the Cu content in the liver[33,40], not something that can be readily measured in human subjects.

Plasma Cu is very tightly regulated and does not correspond to Cu status (liver Cu). Its measurement is made more complex by the fact that most Cu in serum is in the protein ceruloplasmin (Cp), and its regulation may reflect regulation of the protein, rather than of Cu levels.

Proteins binding copper and their role as biomarkers

Many enzymes require Cu for their function, covering a wide range of biological processes. Some of the Cu-requiring enzymes, binding proteins (both intra- or extracellular) and the intracellular Cu chaperones are listed in Table 1. A large number of enzymes need Cu, and many proteins bind and transport Cu. However, no single protein reflects Cu status precisely via its expression level.

Traditional markers of copper status

The traditional approach to the measurement of Cu status has been to use cuproenzymes or total Cu levels in plasma as markers. However, about 95 % of plasma Cu is bound to Cp[41], with the remainder bound by albumin or di-histidine complexes. Cp is an azide-sensitive ferroxidase, and this characteristic enzyme activity is clinically used to estimate plasma Cp levels. It binds between six and eight Cu ions per molecule[42,43]. Cp is generally lower in men than in women[44,45], and is increased by oestrogen, pregnancy and the contraceptive pill. Cp also is an acute-phase reactant, explaining its increase during inflammation, infections and rheumatoid arthritis[46]. Being an acute-phase response protein, Cp increases also in patients with myocardial complications and in cancer patients[47–49]. Further, it is age dependent[50] and subject to seasonal changes[51]. Due to these limitations, Cp cannot be considered a marker for Cu excess[52]. Since serum Cu is a reflection of Cp, the same limitations apply to serum Cu. There is a wealth of data showing that Cp levels decrease in severe Cu deficiency[53]. Cp may, therefore, be a good indicator of Cu deficiency in man, especially when measured from the individual baseline level.

Since Cp levels in plasma remain fairly stable when Cu intake increases, it has been proposed that changes in Cu load may be reflected by the non-Cp Cu fraction. However, the available evidence does not support this idea. The non-Cp fraction cannot be measured directly, and is usually estimated by calculating the amount of Cu contained in Cp (assuming that each Cp molecule contains a mean of six atoms of Cu). This estimated amount is then subtracted from total Cu in the serum. There are several flaws with this approach. The result obtained is a direct function of Cp content in the plasma, with all the implied errors. For example, when non-Cp Cu values are used in clinical practice, a significant number of patients have 'negative values'. This may be caused by errors in Cp measurement or by the variability in Cu atoms bound per Cp molecule, and strongly suggests that non-Cp-bound plasma Cu is not a suitable biomarker for Cu.

Erythrocyte Cu,Zn superoxide dismutase (SOD)-1 has been tested as a biomarker of Cu intake in several studies, but has proven not to be either a reliable or sensitive indicator[53]. Erythrocyte SOD1 activity in a human trial (men) appeared to be influenced by carbohydrate components of the diet, as it was only a good biomarker in a high-fructose diet, but not sensitive to Cu intake if the Cu was fed in a maize-starch diet[54]. Other human studies tested the value of erythrocyte SOD1 activity in men and women, again with negative or equivocal results, for

Table 1. Overview of copper-binding proteins

Protein	Function
Cu-requiring enzymes	
Extracellular	
Cp	Plasma multi-copper oxidase necessary for Fe mobilisation; Cu binding and transport in plasma
Extracellular Cu,Zn SOD3	Involved in defence against reactive oxygen species
PAM	Peptide post-translational activation, modification of many important neuropeptides
Amine oxidases	A group of enzymes oxidising primary monoamines, diamines, and histamine
Lysyl oxidase	Deaminates lysine and hydroxylysine residues in collagen or elastin; involved in formation of cross-links
Intracellular	
CCO	Mitochondrial protein and component of the electron transfer chain
Tyrosinase	Catalyst of melanin and other pigment production
Dopamine β mono-oxygenase	Involved in catecholamine metabolism, catalyses oxidation of 3,4-dihydroxyphenylethylamine to yield noradrenaline
Intracellular Cu,Zn SOD1	Involved in defence against reactive oxygen species)
Phenylalanine hydroxylase	Catalyst of the oxidation of phenylalanine to tyrosine
Hephaestin	Intracellular multi-copper ferroxidase
Cu-binding or -transporting proteins	
Extracellular	
Albumin	Cu binding in plasma
Transcuprein	Cu binding and transport in plasma
Blood clotting factors V and VIII	Blood clotting
Intracellular	
Metallothionein	Cu storage and superoxide scavenging
Glutathione	Metal detoxification
Cartilage matrix glycoprotein	Contributes to the structural integrity of the connective tissue
ATP7A	Cu transporter
ATP7B	Cu transporter
Ctr1	Plasma membrane Cu transporter
Intracellular Cu chaperones	
ATOX1	Delivery of Cu to the Cu ATPase ATP7A (Menkes protein) and ATP7B (Wilson protein)
CCS	Delivery of Cu to SOD1
Cox17	Delivery of Cu to the mitochondria (chaperone for CCO)

Cp, ceruloplasmin; SOD, superoxide dismutase; PAM, peptidylglycine α-amidating mono-oxygenase; CCO, cytochrome C oxidase; Ctr, Cu transporter; CCS, Cu chaperone for superoxide dismutase.

example, a reduction of SOD1 activity in Cu depletion, but no increase in Cu repletion[55], or insensitivity to Cu supplementation[38,52]. Like Cp, SOD1 may indicate Cu deficiency, but is not responsive to excess Cu. A further disadvantage in the assessment of erythrocyte SOD1 is the lack of a standard assay, making clinical measurement and comparison between laboratories difficult. The fact that SOD1 is also an acute-phase reactant and responds to a variety of health and stress conditions probably also contributes to the variable results.

Platelet cytochrome C oxidase (CCO) has been investigated as a biomarker for Cu status in a few studies. Male weanling rats were fed diets containing increasing concentrations of Cu, covering from a range of Cu deficiencies to a Cu-adequate diet. A significant reduction of platelet CCO activity was observed in animals receiving the 3 μg Cu/g diet, correlating with a decrease of Cu levels in the liver[56]. In a study in post-menopausal women fed a diet containing 0·57 mg Cu/d for 105 d, followed by a Cu-repletion period of 2 mg Cu/d for 35 d, CCO was the most sensitive marker, as compared with plasma Cu, Cp or erythrocyte SOD1. Platelet CCO activity dropped to 49 % during Cu depletion, then increased back to 60 % of the control level (entry level) during Cu repletion[55]. It is currently unclear whether an increase in Cu status is paralleled by an increase in platelet CCO activity. CCO is a labile enzyme, displaying large inter-subject variation, factors that may limit its use in the field[31].

Plasma diamine oxidase (DAO) activity was reported to be decreased in rats fed marginal- and low-Cu diets[57]. This decline was parallel to a drop in liver Cu, while other cuproenzymes remained unaltered. Reduced plasma DAO activity was also found in Cu-deficient human subjects and in long-term Cu-deficient rats[58]. These findings suggest plasma DAO may be a very sensitive marker for marginal to marked Cu deficiency. An increase in plasma DAO activity, however, is indicative of tissue injury and used clinically for the detection of injury of intestinal tissue[59,60], excluding it from use as a wide-range indicator of Cu status. Nonetheless, it may have value as part of a suite of markers.

Liver aminotransferases are traditionally used as biomarkers for high Cu status, since Cu is deposited in the liver, and excess Cu results in liver tissue damage. In a recent trial, Cu supplementation of 10 mg/d was given for 60 d to a study population with naturally high or low serum Cp. It was shown that the liver aminotransferases glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase and γ-glutamyltransferase had increased in both groups in response to elevated Cu intake after the 2-month loading period was completed. While the increase was significant, liver aminotransferases remained below the clinical cut-off level to indicate liver pathology. This increase was transient, and not associated with any liver dysfunction, indicating that these markers may be useful in detecting excess Cu intake within the subgroups carrying Cu-related polymorphisms (resulting in a high- or low-Cp phenotype). Further studies to characterise these subgroups are clearly needed[52].

In a similar trial on subjects with normally distributed serum Cp levels, study participants ingested 2 to 6 mg Cu/l in drinking water. Gastrointestinal symptoms increased in the Cu-supplemented groups in a dose-dependent manner[61], which was interpreted as a consequence of acute Cu exposure. However, there was no change in liver enzyme levels in the serum. Further, since none of these liver enzymes respond to Cu deficiency, they cannot be considered as reasonable candidates for full-range biomarkers for Cu in a normal population.

A plasma cuproenzyme with potential to be used as a biomarker for Cu is peptidylglycine α-amidating mono-oxygenase (PAM), the advantage being that a small amount of blood from a finger-prick sample may be sufficient to detect changes. In individuals suffering from genetic Cu deficiency (Menkes' disease and occipital horn syndrome), plasma PAM is Cu depleted, leading to a decrease in enzyme activity. *In vitro* addition of Cu to blood samples from these patients repletes PAM activity. This test shows an increased Cu stimulation index of PAM in mild to severe Cu deficiency, when compared with healthy controls[62]. PAM activity has been shown to be generally lower in various models of Cu deficiency in rodents[63]. PAM was suggested to be less sensitive to endocrine changes than, for example, Cp, and may be a useful marker for Cu deficiency during development and childhood[63]. It has to be kept in mind that PAM has so far only been tested in Cu deficiency, and its activity is unlikely to increase in parallel with elevated Cu.

Lysyl oxidase is a cuproenzme involved in the formation of cross-links in collagen and elastin. Hence, high concentrations of lysyl oxidase are found in connective tissues, such as tendon and skin[64]. The functional activity of the enzyme in tissue is decreased in dietary Cu deficiency[64–67], probably as a result of post-translational processing (Cu incorporation) of the enzyme[68]. In parallel, increased dietary Cu causes activation of lysyl oxidase in chicken tendon[69]. Despite these promising findings, the tissue expression pattern (connective tissue) of lysyl oxidase is not suited for convenient accessibility. Its use as a biomarker in man is limited.

Expression of proteins, such as metallothionein in the liver[70] and Cu transporter-1 in several organs[71], has been shown to be regulated by Cu status. However, their value as a biomarker is limited since they are located within organs that are not readily accessible. If these candidates prove to be regulated also in accessible tissues (blood erythrocytes and monocytes, buccal cells), and if sufficiently sensitive and reliable assays can be established, proteins expressed highly in liver may serve as a biomarker for Cu.

Non-traditional biomarkers of copper status

Some components of the immune system have been suggested as a potential biomarker of marginal Cu deficiency. IL-2, but not TNF-α, was shown to increase in response to Cu supplementation in individuals with naturally low plasma Cp levels[72]. This suggests that IL-2 may be an identifier of a sub-population with a low-Cp phenotype, and may serve as a biomarker for elevated Cu in this subgroup. Many immune markers (IL-2 production, neutrophil function, phenotypic profiling of lymphocyte subsets and the blastogenic response to T cell mitogens) have been shown to be sensitive to mild Cu deficiency, and, what is more, Cu repletion has in many cases restored their function to control levels (for a review, see Bonham *et al.*[12]). The observation that *in vitro* activity of T lymphocytes and neutrophils is suppressed by low Cu intake in adult male rats, but not in females, suggests the need for further investigation of immune markers in the whole population[73].

Another potential marker of a mild decrease in Cu intake is bone metabolism. A dietary intervention study in adult healthy men showed that urinary pyridinoline and deoxypyridinoline, both biomarkers of bone resorption, significantly increased when the volunteers' diet was switched from medium (1·6 mg Cu/d) to low (0·7 mg Cu/d) intake. Conversely, the urinary bone resorption markers decreased when Cu intake was elevated from low to high (6 mg Cu/d) levels. The volunteers were exposed to the experimental diets for 8 weeks. Serum Cp was unaffected in this study[74]. These findings, however, were not confirmed in a similar study in females, where Cu supplements of 0, 3 or 6 mg Cu/d during 4 weeks did not affect urinary bone markers[75]. This discrepancy may be explained by that fact that bone metabolism responds to several dietary and other factors, such as vitamin D levels and sunlight. The variety of factors influencing bone resorption makes the interpretation of changes in those markers difficult. While undoubtedly influenced by dietary Cu, bone markers may be too complex to be useful as a Cu-specific biomarker.

Cu deficiency causes an elevation in blood lipoproteins[13,76–79]. Despite these findings, blood lipoprotein metabolism is not a promising biomarker for Cu status, as too many other factors, such as diet and lifestyle, affect these parameters.

Copper chaperones

The discovery of the first Cu chaperone in 1995 has revolutionised our understanding of intracellular Cu trafficking[80]. After Cu is transported across the plasma membrane, intracellular Cu is carried via specific chaperone proteins to Cu-dependent enzymes directly, or to compartments in which Cu-dependent enzymes are matured. The three Cu chaperones identified to date, and conserved from yeast cells to man, are listed in Table 1. Cox17 delivers Cu to the mitochondria, where it is ultimately incorporated into CCO. The Cu chaperone for SOD (CCS) delivers Cu to the metal-binding site of Cu,Zn-SOD in the cytosol and in the mitochondrial inter-membrane space. Atox1 delivers Cu to the two mammalian P-type Cu-transporting ATPases, ATP7A and ATP7B. This ensures 'safe' Cu trafficking, and is the basis for the observation that, in a healthy cell and in a physiological environment, virtually no Cu is unbound. In order to ensure safe intracellular Cu delivery, chaperones need to respond to Cu exposure in a very sensitive way. Chaperones are not present in plasma; however, their expression in blood erythrocytes and white cells offers a possibility for easy-access measurement.

The most promising candidate for accurate and sensitive Cu status detection is the CCS. In a rat feeding study, CCS was reported to be responsive to Cu-marginal and Cu-deficient diets. CCS expression was measured with a specific antibody (Western blot) and was shown to increase in a dose–response fashion with a decrease in Cu intake in liver as well as in blood erythrocytes[81]. CCS was also shown to respond to mild secondary Cu deficiency (induced by Zn supplementation) in rats[82]. These finding are confirmed by a study in various models of Cu deficiency in mice and rats, where CCS protein was demonstrated to be consistently elevated in Cu deficiency[83,84]. This was concomitant with a decrease in SOD1 level, and the authors suggested using erythrocyte CCS:SOD1 for the determination of Cu deficiency.

Until recently, it was unclear whether CCS is negatively regulated by excess Cu[85]. In a recent study, CCS expression was measured by real-time RT-PCR in peripheral mononuclear cells isolated from healthy men that were supplemented with 8 mg Cu/d for 6 months. Peripheral mononuclear cell CCS mRNA transcripts decreased significantly in the Cu-treated group after 6 months, strongly suggesting that this may be a useful indicator of Cu exposure (M Suazo and M Araya, unpublished results). A similar protocol was applied to a subpopulation of healthy adults, representing the 5 % highest and lowest extremes in the serum Cp concentration distribution curve. Peripheral mononuclear cells were isolated from study participants before and after exposure to a single daily does of 10 mg Cu for 2 months. No change in CCS expression was observed in the low-Cp phenotype, but in the high-Cp individuals, CCS was decreased significantly in response to the Cu supplementation (M Suazo and M Araya, unpublished results). These data, along with other studies of CCS in animals and cell lines with Cu deficiency, support the idea that CCS is the most promising potential marker for deficiency as well as excess states of Cu, and call for its further characterisation.

Outlook

In summary, while some blood markers may indicate moderate and severe Cu deficiency (Cp, erythrocyte SOD1, PAM, DAO) there is no good marker for Cu excess, even at a level where symptoms such as acute nausea and abdominal pain are reported[34,61].

Many of the potential markers of Cu status listed in Table 1 have not been tested yet. Rather than testing each of the cuproenzymes, Cu-binding proteins or Cu chaperones for potential use as a Cu biomarker, the advent of high-throughput technologies has made it possible to screen for potential biomarkers in the whole proteome of a cell. This 'non-hypothesis-driven search' has the advantage of not excluding markers that have no direct connection to Cu. Further, the screening allows us to search for a group of proteins that, in combination, are reflective of Cu status. Since Cu is involved in so many biological processes, a good biomarker may be a very downstream product, with no immediate role in Cu metabolism. This non-exclusive and open approach may allow for the determination of a novel type of biomarker: the biomarker suite.

The biomarker suite opens the door to another possibility: it may be appropriate to develop different groups of sensitive biomarkers for different population subgroups. Hypothetically, this may lead to a suite of Cp, immunological markers and bone indicators only for adult males[73–75]. For example, a suite may contain metallothionein for use only in adults, but not neonates[86]. PAM, in turn, may be introduced in a suite for Cu status during development[63]. Finally, we expect that this approach may be extended to the examination of other nutritional states arising as a consequence of inappropriate nutrition.

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